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Amino Acid Sequence in All the Tryptic Peptides from the α Polypeptide Chain of AII Component of Chicken Hemoglobin*

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Hemoglobin was obtained from blood of adult white leghorns. It was first separated into three components, AI, AII, and AIII by CMC-column chromatography. AII, the greatest component of the three was chosen as the subject matter of the present study. It was first converted into globin by removal of heme in acetone-HCl solution, and then the globin was separated into α and β polypeptide chains by Amberlite CG50 column chromatography. The α polypeptide chain was digested with trypsin, and tryptic peptides thus obtained were subjected to column chromatography and then to paper chromatography for isolation and purification. The column chromatography was carried out by using Dowex 1X2 as the adsorbent and the acetate buffer containing organic bases such as pyridine, collidine, lutidine, and picoline as the developer. The developer for the paper chromatography was the mixture of n-butanol, acetic acid, and water.

The amino acid composition of the tryptic peptides thus isolated and purified was analyzed on an amino acid analyzer and subsequently the amino acid sequence in these tryptic peptides was determined by using partial hydrolyses with various enzymes, the PTC method, and the DNP method. The results were discussed in comparison with human hemoglobin. As a result, replacement of amino acids in the tryptic peptides between adult human and white leghorn hemoglobins was presumed at 35 positions in the α polypeptide chain.

INTRODUCTION

With the recent progress of protein chemistry, attempts to elucidate problems on evolution and phylogeny of living organisms on molecular level have extensively been made since 1950. Above all, since amino acid sequence in protein molecules, i. e. the primary structure of proteins was known to be a precise copy of the hereditary message of DNA in cell nucleus, a great importance has been placed on the

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comparative studies on the primary structures of protein molecules from living organisms belonging to different species because they give some clues for phylogeny.

According as the advance in theories of protein chemistry and analytical techniques prompt many groups of workers to uudertake investigations on the primary structure, information on the correlation between the primary structure and its biological function was also derived. It was confirmed that the two living organisms belonging to considerably remote species possess some kinds of protein whose primary structure as well as biological function is remarkably similar to each other, and this fact elicited a hypothesis that such proteins are due to a common ancestral gene possessed by the both.

On the other hand, it is generally presumed that in proteins with the biological activity, a certain part of the primary structure plays a much more important role than other parts, and therefore, if the amino acid sequence in this important part is unchanged, proteins do not lose their function even though there exist some differences in the amino acid sequence of other parts.

For promoting the studies as stated above, hemoglobin is one of the most useful experimental materials not only because it can be found very commonly in the animal kingdom but because it can be isolated and purified relatively more readily than other proteins. Since BRAUNITZER et al.[9] and KONIGSBERG et al.[24] established the primary structure of human hemoglobin, many groups of workers in the world have studied[8),13),44),52] or are studying[4),38),40),45] on the primary structure of mammlian hemoglobins from various species. In 1968, MATSUDA et al.[31] of our department determined the whole primary structure of adult rhesus monkey hemoglobin. With respect of non-mammalian hemoglobins, however, BRAUNITZER et al.[21] only reported the determination of the primary structure of the α polypeptide chain of carp hemoglobin, and no reports have been presented on avian hemoglobins.

Our department, with an interest in the phylogenetic relationship between human and Chicken hemoglobins as well as their resemblance in function such as Bohr's effect and the oxygen dissociation curve, have been investigating on the primary structure of adult hemoglobin of the Chicken.

As a first step, the present author determined the amino acid sequence in all the tryptic peptides from the α polypeptide chain of the greatest component, AII of adult Chicken hemoglobin and compared the results with the known sequence of human hemoglobin.

MATERIALS AND METHODS

1) *Preparation of Hemoglobin from Adult Chickens.*

Hemoglobin solution was obtained by DRABKIN's method[17] from

blood of adult pure-line white leghorns (Smal A type) as follows.

Blood was obtained from the jugular vein of white leghorns, and to this was added 3.8% sodium citrate solution as an anticoagulant. The blood was then submitted to centrifugation in order to remove plasma and then to this was added an equal volume of deionized water and half the volume of toluene. The mixture was hemolyzed overnight in the cold (2° to 4°). The hemolysate was centrifuged at 12,000 r.p.m. for 1 hour at 0°, and the hemoglobin solution was obtained.

2) *Isolation of AII Component from the Hemoglobin Solution.*

AII component was isolated from the hemoglobin solution according to MATSUDA et al.[32]

The activated carboxymethyl cellulose(Brown Company, 0.71 mEq/g) was poured into a column (2×30 cm), which was equilibrated with 0.01 M phosphate buffer, pH 6.9, The hemoglobin solution, 5 ml, was placed on the column. The linear gradient elution was carried out by means of phosphate concentration. The fraction volume was 25 ml. The optical density of the effluent was measured at 542 mμ and 280 mμ. Fractions at each peak were combined, dialyzed against deionized water, and finally lyophilized.

3) *Preparation of Globin.*

Removal of heme from AII component was performed according to ANSON and MIRSKY.[2] The lyophilized AII component, about 5,000 mg, was added dropwise with stirring to HCl-acetone solution cooled at -20°. The solution was centrifuged at 3,000 r.p.m. for 5 min at 0° to 4°, dissolved in an appropriate volume of deionized water, dialyzed against deionized water, and finally lyophilized.

4) *Isolation of α Polypeptide Chain from the Globin of AII Component.*

The globin obtained from AII component was separated into α and ß polypeptide chains by the method of SMITH[50] and MATSUDA et al.[29] The column (3.3×45 cm) was prepared with the activated Amberlite CG50 (Type II, H-form) and then equilibrated with 3N formic acid. To this column was applied 1,000 mg of the globin from AII component. The linear gradient elution was performed by means of urea concentration. The fraction volume was 25 ml. The effluent was analyzed by optical density at 280 mμ. Fractions at each peak were combined, dialyzed against deionized water, and then lyophilized.

5) *Determination of the N-Terminal Structure of the α Polypeptide Chain by the DNP Method.*

The DNP method was carried out as described by MATSUDA et al.[30]

The α polypeptide chain, 100 mg, was dinitrophenylated and DNP-peptides thus obtained were hydrolyzed with constant boiling HCl and extracted with ether. The extracts were placed on a silica gel-Celite

column (1×15 cm). Development was performed with the mixture of acetic acid, acetone, and ligroin.

6) Determination of Cysteine Residues in the α Polypeptide Chain.

Estimation of cysteic acids derived from cysteine residues in the α polypeptide chain was carried out according to MOORE[33].

The α polypeptide chain, 5 μmoles (85 mg), was oxidized with performic acid by the method of HIRS[22], and then hydrolyzed with constant boiling HCl for 18 hours. The hydrolysate was placed on a column (0.9×50 cm) of the activated Amberlite CG 120 equilibrated with 0.2 N sodium citrate buffer, pH 3.25. Elution was carried out by using 0.2 N sodium citrate buffer, pH 3.25, as a developer. Analysis of the effluent was performed both by the ninhydrin reaction according to MOORE et al.[34] and by measuring the optical density at 570 mμ.

7) Determination of Tryptophan Residues in the α Polypeptide Chain.

Estimation of tryptophan was carried out according to KOSHLAND et al.[3].

The α polypeptide chain, 10 μmoles (170 mg), dissolved in 10 M urea was incubated at 37° for 16 hours, and to this was added HNB-bromide. The α polypeptide chain thus treated was placed on a column (1.0×23 cm) of the activated Sephadex G-25 equilibrated with 10 M urea, pH 2.7. Elution was carried out with the developer, 10 M urea, pH 2.7. The fraction volume was 2 ml. Optical density of the effluent was measured at 410 mμ.

8) Digestion of the α Polypeptide Chain with Trypsin.

The α polypeptide chain, 1,500 mg, was denatured by the method of BRAUNITZER et al.[26] and then digested at 37° for 4 hours by the addition of trypsin (Worthington Biochemical Co., twice crystallized) prepared according to REDFIELD et al.[39] and BRAUNITZER et al.[14]. After the termination of the digestion, the digest was adjusted to pH 6.1. Then, the so-called insoluble tryptic peptide was precipitated. The digest was submitted to centrifugation so that it was separated into the soluble tryptic peptides and the insoluble tryptic peptide, both of which were lyophilized, respectively.

9) Column Chromatography of the Soluble Tryptic Peptides.

MAITA's method[27] was used. The activated Dowex 1X2 (200 to 400 mesh, Dow Chemical Co.) was poured in a column (0.9×150 cm), which was equilibrated with 1% pyridine, 1% collidine acetate buffer, pH 9.0. The soluble tryptic peptides dissolved in deionized water were placed on the above-prepared column. The pH linear gradient elution was carried out by using both pyridine, 2-4 lutidine, α-picoline acetate buffer and acetic acid; Fraction No.1 to 20 were eluted with the starting buffer, 1% pyridine, 1% collidine acetate buffer, pH 9.0 ; for

Fraction No.21 to 40, the buffer was changed stepwise into 1% pyridine, 1% collidine acetate buffer, pH 8.5 ; Fraction No. 41 to the fraction at pH 5.5 were eluted by supplying 0.1 N acetic acid in the upper chamber to the mixing chamber containing 1,500 ml of 1% pyridine, 1% picoline, 1% 2-4 lutidine acetate buffer, pH 7.5 ; To the fraction the effluent of which lowered to pH 3.5, 1 N acetic acid was supplied, and finally glacial acetic acid was used.

An aliquot of each fraction after the alkali-hydrolysis was submitted to the ninhydrin reaction according to the method of YEMM and COCKING[51], and then optical density was measured at 570 mμ. Fractions at the peaks were combined respectively and dried below 37° under reduced pressure on a rotary evaporator. Each of the dried samples was dissolved in 3 ml of deionized water. Some peptides insoluble in water were dissolved in 50% acetic acid solution by the final acetic acid concentration of 12 to 13%[6].

10) Digestion of the So-Called Insoluble Tryptic Peptide with Pepsin.

The insoluble tryptic peptide was oxidized with performic acid by the method of HIRS[22]. Digestion was carried out at 37° for 2 hours by adding pepsin (Sigma Chemical Co., Three time crystallized) by a ratio of 1% to the substrate. At the termination of the digestion, the digest was adjusted to pH 10.0 and the dried under reduced pressure below 37°. Column chromatography for the insoluble tryptic peptide was carried out in the same way as for the soluble tryptic peptides.

11) Digestion of the Peptides, α TVI-b, α TVII-a, and α TIX-a with Pepsin.

Each of these peptides, about 10 μmoles, was dissolved in deionized water,and to this was added HCl solution containing pepsin (Sigma Chemical Co., three time crystallized). α TVI-b, α TVII-a, and α TIX-a were digested at 37° for 6 hours, 4 hours, and 16 hours, respectively. The digests were adjusted to pH 10.0 and dried under reduced pressure.

A column (0.9×60 cm) was prepared with the activated Dowex 1 X2 (200 to 400 mesh) and equilibrated with 1% pyridine, 1%2-4 lutidine acetate buffer, pH 8.5. To this column was applied the above-prepared peptic peptides, and then development was performed at a flow rate of 60 ml per hour by using the pH linear gradient elution with both 1% pyridine, 1% 2-4 lutidine acetate buffer and acetic acid; Fractions No. 1 to 15 were eluted with the starting buffer, 1% pyridine, 1% 2-4 lutidine acetate buffer; until the pH of the effluent lowered to 6.0, 0.1 N acetic acid was supplied from the upper chamber to the mixing chamber containing 700 ml of the starting buffer; until the pH of the effluent lowered to 3.5, 1 N acetic acid was supplied and finally glacial acetic acid was used. During the development, the column was kept at 37°. The effluent was collected into 8 ml-fractions by a fraction collector.

An aliquot of each fraction was submitted to the ninhydrin reaction[51] and optical density was measured at 570 mμ. Fractions at each peak were combined together, dried under reduced pressure below 37°, and then dissolved in 3 ml of deionized water.

12) Paper Chromatography of the Tryptic Peptides and the Peptic Peptides from the So-Called Insoluble Tryptic Peptide and α TVI-b, α TVII-a, and α TIX-a.

The sample, 50 μl, was applied as a spot on a sheet of Toyo filter paper No. 50 (60×60 cm). Development was carried out at 25° in the constant temperature room by the descending method with the upper phase of the mixture of n-butanol, acetic acid, and water (4 : 1 : 5, v/v)[36].

Peptides were detected by spraying 0.2% ninhydrin-n-butanol solution on the paper and then heating to color with an iron. The EHRLICH reaction[46], the SAKAGUCHI reaction[23], the PAULY reaction[47], and the α-nitrosonaphtol reaction[1] were also performed on the paper.

Purification of peptides by paper chromatography was performed as follows; Peptides were detected with 0.02% ninhydrin-n-butanol solution and then eluted with 5% acetic acid solution for 16 hours at the room temperature. The eluate was dried under reduced pressure.

13) The PTC Method.

A modification of EDMAN's original method[24] was employed for phenylisothiocyanation of the peptides. The ring-formation was performed by using trifluoroacetic acid (the special grade). Analysis was carried out by the degradation method.

The peptides were dissolved in 2.0 ml of 66% pyridine solution contained in a heart-shaped flask. To this was added 0.05 ml of phenylisothiocyanate (PTC). The mixture was allowed to react at pH 7.5 and 37° for 2 hours and then dried under reduced pressure below 37°. The flask was connected to the cold finger condenser and evacuated at 40° for 40 min in a water bath by a vacuum pump. To this was added 1 ml of trifluoroacetic acid. The mixture was allowed to stand for 4 hours at the room temperature, dried under reduced pressure below 37°. It was dissolved in deionized water and extracted three times with 5 ml of benzene. An aliquot of the aqueous phase dried under reduced pressure was hydrolyzed with constant boiling HCl at 105° for 20 hours, and the amino acid composition of the hydrolysate was analyzed.

The remainder of the aqueous phase was dried under reduced pressure and used for the next stage of the PTC method.

14) N-Terminal Analyses of the Peptides by the DNP Method.

LEVY's method[25] was employed. The peptides were dinitrophenylated at 40° for 2 hours in 1% $NaHCO_3$ solution. After the reaction

stopped, the excess of 1-fluoro-2, 4-dinitrobenzene (DNFB) was removed by ether extraction. The aqueous was dried under reduced pressure and then hydrolyzed with constant boiling HCl at 105° for 20 hours. N-terminal DNP-amino acid was extracted with ether and identified by the two dimensional paper chromatography. Developers were n-butanol saturated with 1 N NH_4OH for the first dimension and 0.5 M phosphate buffer for the second. Quantitative analysis of the DNP-amino acid was performed in the following way; Yellow spots on the paper chromatogram were cut out and extracted with 5 ml of warm water, respectively. The optical density of the eluate was measured at 360 mμ.

15) Peper Electrophoresis of the Peptides.

In order to know electric charge of the peptides and to distinguish acidic amino acid from their amides, high voltage paper electrophoresis was carried out at 2 KV for 2 hours on an electrophoretic apparatus (Ishidai type), the sample being applied on the center of a sheet of Toyo filter paper (10×60 cm), No. 50. Pyridine acetate buffer, pH 6.4 (pyridine : acetic acid : water = 100 : 4 : 900, v/v) was used as a developer[41]. After the paper was dried in the air, peptides were detected by spraying 0.2% ninhydrin-n-butanol solution and then heating to color with an iron.

16) Amino Acid Analyses of the Peptides.

The purified peptides dissolved in constant boiling HCl were hydrolyzed at 105° for 20 hours in a sealed tube. The hydrolysate was dried under reduced pressure, and then the amino acid composition was analyzed on Hitachi KLA-2 amino acid analyzer.

RESULTS

1) Isolation of AII Component from Hemoglobin Solution.

Fig. 1 shows three peaks, which were designated AIII, AI, and AII in the elution order. Their yields were 4.8%, 23.8%, and 71.4%, respectively. The recovery was 87%. MANWELL[28] also obtained almost the same results.

2) Isolation of α and β polypeptide chains

The yield of the α polypeptide chain was 44% and that of the β polypeptide chain 56%. The recovery was about 60%.

3) Determination of the N-Terminal Structure of the α Polypeptide Chain by the DNP Method.

The N-terminal analysis of the α polypeptide chain resulted in 0.53 mole of DNP-Val, 1.73 mole of DNP-Val-Leu, and none of DNP-Val-His, indicating that the α polypeptide chain had been isolated almost in purity as previously reported by MATSUDA et al.[31]

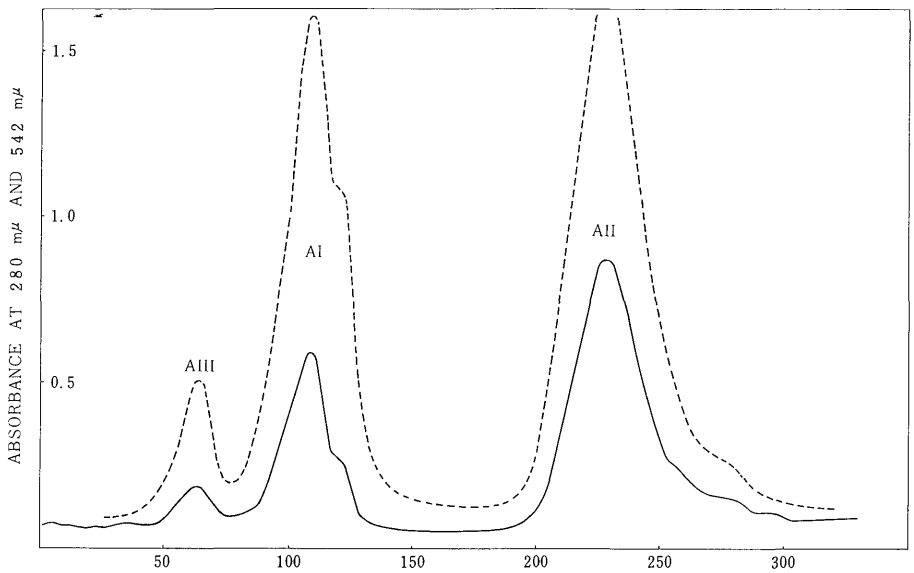


Fig. 1. Column chromatogram of adult chicken hemoglobin.
CM-Cellulose column : 2 x 30 cm.——Absorbance at 542 mμ.·····Absorbance at 280 mu.

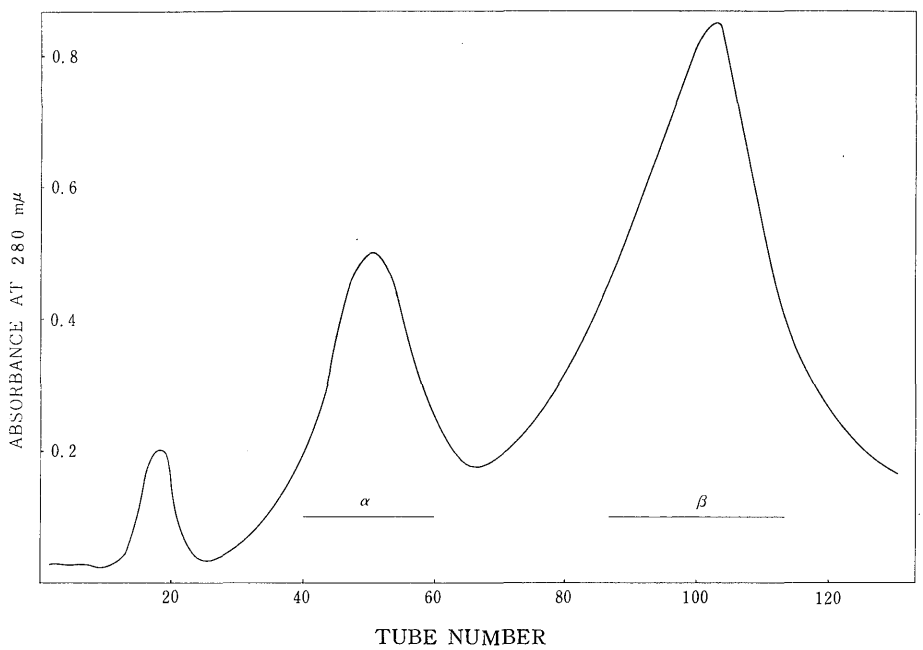


Fig. 2. Separation of globin from AII Component into α and β subunits. Column: IRC-50 (Type II), 3.3 x 45 cm.

4) *Determination of Cysteine Residues as Cysteic Acid in the α Polypeptide Chain.*
Estimation of cysteic acid resulted in 1.40 mole per one molecule of the α polypeptide chain.

5) *Determination of Tryptophan Residues in the α Polypeptide Chain.*
Estimation of tryptophan resulted in 0.14 mole per one molecule of the α polypeptide chain.

6) *Column Chromatography, Paper chromatogrophy, and Amino Acid Composition of the Soluble Tryptic Peptides from the α Polypeptide Chain.*
Nine peaks were obtained as shown in Fig. 3.

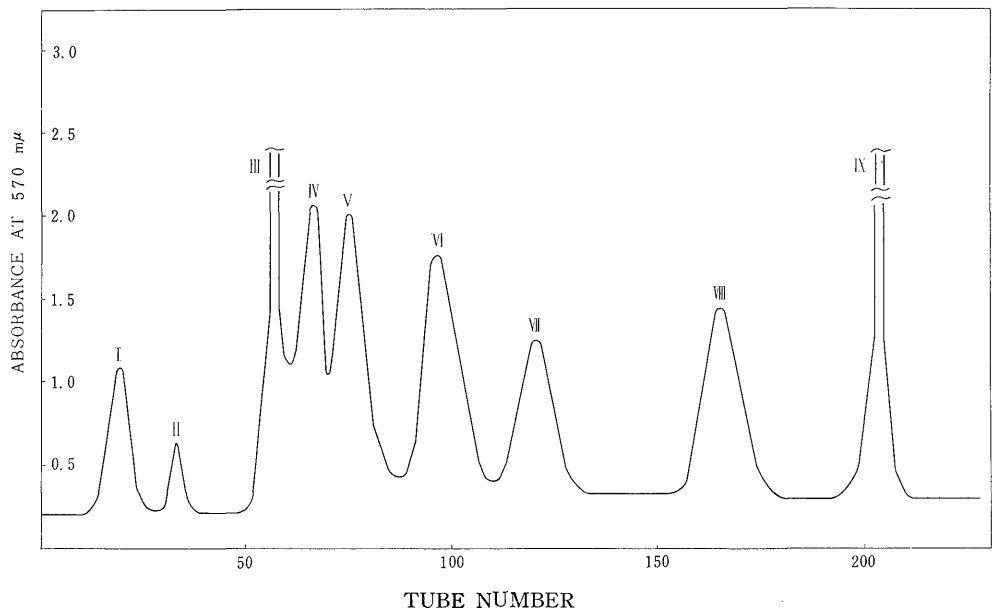
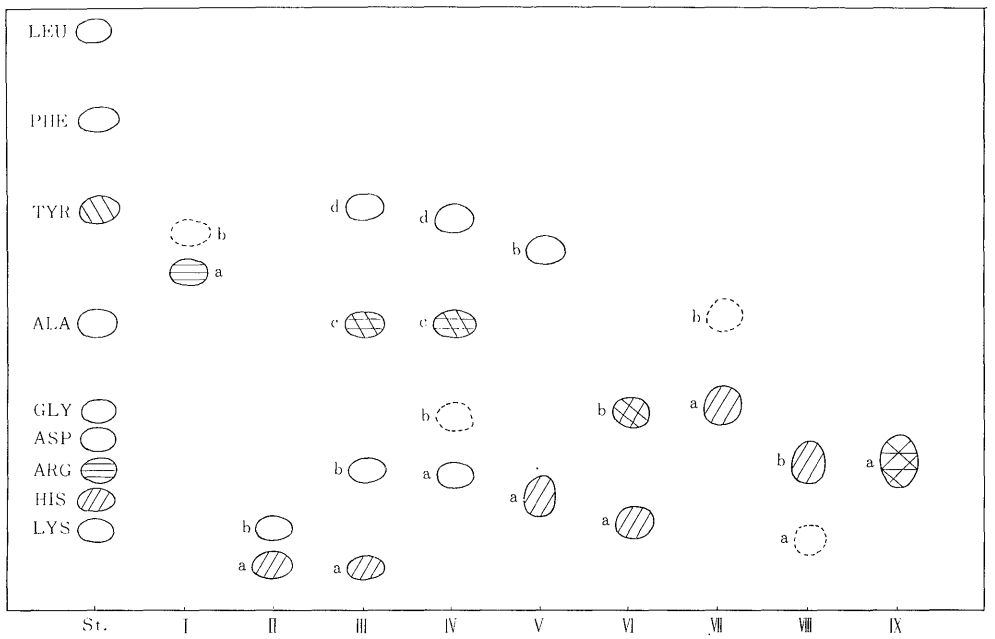


Fig. 3. Separation of the tryptic peptides from the α polypeptide chain of AII component of adult chicken hemoglobin.

Each peak was submitted to paper chromatography. The result was given in Fig. 4, where 17 main spots were observed. Four of these spots were positive to the SAKAGUEHI reaction ; seven spots positive to the PAULY reaction ; four spots positive to the α-nitrsonaphtol reaction and none positive to the EHRLICH reaction. Table I gives the amino acid composition of these spots. α TII-a and α TIII-a, α TIII-c and α TIV-c were identical in the amino acid composition, respectively.

7) *Sequence Analyses of the Soluble Tryptic Peptides from the α Polypeptide Chain.*
Amino acid sequence in each tryptic peptide was analyzed by using the DNP method and the PTC method together.

(1) α TI-a (Rf Leu 0.59)
The N-terminal residue was known to be leucine by the DNP met-



PEAK NUMBER IN FIG. 3

Fig. 4. Paper chromatogram of the tryptic peptides from the α polypeptide chain of AII Component of adult chicken hemoglobin.

Table I Amino acid compositions of the tryptic peptides from the α polypeptide chain of AII Component of adult chicken homoglobin

	Ia	IIa	IIb	IIIb	IIIc	IIId	IVa	IVd	Va	Vb	VIa	VIb	VIIa	VIIIb	IXa
Lys		1.03	1.00	1.02		1.02	1.01	1.08	0.98	1.07	1.06	1.12	1.92	0.98	0.93
His		1.02							2.03		0.94	1.88	1.08	1.02	1.06
Arg	0.90				1.09										
Cys															
Asp				2.08			2.26		1.12	2.17	1.18	1.09	2.88	2.81	
Thr						2.03		1.02				0.93	0.90	0.90	0.94
Ser							0.86		0.88		0.71	1.83	1.74	1.82	
Glu												1.03	1.07	1.01	4.01
Pro						0.71				0.96		0.95			
Gly		2.03				1.21		1.08				1.10	0.97	0.98	1.96
Ala							1.16		1.10		0.97	1.21	4.93	4.84	3.00
Val				0.92			0.92			1.98	0.63		1.15	0.91	
Met						+									
Ileu						1.15		0.95				0.91	2.96	2.93	0.94
Leu	1.10						0.78		1.90		0.98	1.14	2.08	2.02	1.05
Tyr					0.91							0.72			0.76
Phe						0.81		0.94		0.88		1.65			

hod. The sequence is Leu-Arg. D indicates the DNP method hereafter.

$$\overrightarrow{D}$$

(2) α TII-a (Rf Leu 0.07)

The following result was obtained by the PTC method.

		Lys	His	Gly
composition		1.03	1.02	2.03
stage	1	1.00	0.92	1.08
	2	1.00	0.30	1.00

The squence is, therefore, Gly-His-Gly-Lys. P indicates the PTC method hereafter.

$$\overrightarrow{P}\ \overrightarrow{P}$$

(3) α TII-b (RfLeu 0.18)

It was confirmed to be a single lysine by the descending paper chromatography and amino acid analyzer.

(4) α TIII-b (Rf Leu 0.24)

The following result was obtained by the PTC method.

		Lys	Asp	Val
composition		1.02	2.08	0.92
stage	1	n.d.	1.17	0.83
	2	n.d.	0.66	1.00

n.d. indicates "not determined", hereafter. The sequence was therefore determined to be Asp-Asp-Val-Lys.

$$\overrightarrow{P}\ \overrightarrow{P}$$

Distinction between acidic amino acids and their amides was hereafter performed by paper electrophoresis.

(5) α TIII-c (Rf Leu 0.50)

The N-terminal amino acid turned out to be tyrosine by the DNP method. The sequence was Tyr-Arg.

$$\overrightarrow{D}$$

(6) α TIII-d (Rf Leu 0.71)

Analysis by the PTC method resulted as follow;

		Lys	Thr	Pro	Gly	$MetO_2$	Ileu	Phe
Composition		1.02	2.03	0.71	1.21	+	1.15	1.81
stage	1	n.d.	2.18	0.86	0.88	−	1.22	1.83
	2	n.d.	2.10	0.93	0.86	−	1.03	0.84
	3	n.d.	2.09	1.08	0.91	−	0.07	0.91
	4	n.d.	2.08	0.97	0.02	−	0.00	0.91
	5	n.d.	2.08	0.93	0.00	−	0.00	0.12
	6	n.d.	2.00	0.23	0.00	−	0.00	0.00

The sequence was, therefore, Met-Phe-Ileu-Gly-Phe-Pro-Thr-Thr-Lys.

→ → → → → →
P P P P P P

(7) αTIV-a (Rf Leu 0.22)

The following result was obtained by the PTC method.

		Lys	Asp	Ser	Ala	Val	Leu
composition		1.01	2.26	0.86	1.16	0.92	0.78
stage	1	n.d.	2.04	0.86	1.18	**0.00**	0.78
	2	n.d.	2.12	0.84	1.04	0.00	**0.00**
	3	n.d.	1.85	**0.08**	1.07	0.00	0.00
	4	n.d.	**1.02**	0.00	0.98	0.00	0.00
	5	n.d.	1.00	0.00	**0.00**	0.00	0.00

The sequence was determined to be Val-Leu-Ser-Asn-Ala-Asp-Lys

→ → → → →
P P P P P

(8) α TIV-d (Rf Leu 0.68)

Analysis by the PTC method gave the following result.

		Lys	Thr	Gly	Ileu	Phe
composition		1.08	1.02	1.08	0.95	0.94
stage	1	n.d.	1.07	**0.00**	0.98	0.95
	2	n.d.	0.92	0.00	**0.00**	1.08
	3	n.d.	1.00	0.00	0.00	**0.12**

The sequence was therefore determined to be Gly-Ileu-Phe-Thr-Lys.

→ → →
P P P

(9) αTV-a (Rf Leu 0.20)

The following result was obtained by the PTC method.

		Lys	His	Asp	Ser	Ala	Leu
composition		0.98	2.03	1.12	0.88	1.10	1.90
stage	1	n.d.	n.d.	1.21	0.81	1.11	**0.90**
	2	n.d.	n.d.	1.21	**0.18**	1.07	0.73
	3	n.d.	n.d.	**0.35**	0.05	1.00	0.78
	4	n.d.	n.d.	0.00	0.00	1.00	**0.18**
	5	n.d.	**n.d.**	0.00	0.00	1.00	0.00
	6	n.d.	n.d.	0.00	0.00	**0.28**	0.00

The sequence was therefore Leu-Ser-Asp-Leu-His-Ala-His-Lys.

→ → → → → →
P P P P P P

(10) αTV-6 (Rf Leu 0.61)

It was analyzed by the PTC method as follows.

		Lys	Asp	Pro	Val	Phe
composition		1.07	2.17	0.96	1.98	0.88
stage	1	n.d.	2.05	0.86	**1.06**	1.00
	2	n.d.	**1.24**	0.79	0.95	1.01

3	n.d.	1.14	**0.16**	0.97	0.88
4	n.d.	1.15	0.00	**0.13**	0.85
5	n.d.	**0.38**	0.00	0.00	1.00

The sequence was therefore Val-Asp-Pro-Val-Asn-Phe-Lys.

→ → → → →
P P P P P

(11) αTVI-a (Rf Leu 0.16)

The following result was obtained by the PTC method.

	Lys	His	Asp	Ser	Ala	Val	Leu
composition	1.06	0.94	1.18	0.71	0.97	0.63	0.98
stage 1	n.d.	n.d.	1.15	0.84	1.07	**0.00**	0.85
2	n.d.	**n.d.**	1.16	0.95	0.90	0.00	0.98
3	n.d.	n.d.	1.10	0.88	**0.08**	0.00	0.96
4	n.d.	n.d.	1.08	**0.02**	0.00	0.00	0.90
5	n.d.	n.d.	1.00	0.00	0.00	0.00	**0.10**

The sequence was determined to be Val-His-Ala-Ser-Leu-Asp-Lys.

→ → → → →
P P P P P

(12) α TVI-b (Rf Leu 0.32)

This peptide was digested with pepsin and chromatographed on a column (0.9 x 60cm) of Dowex 1X2. As shown in Fig. 5, two peaks were obtained.

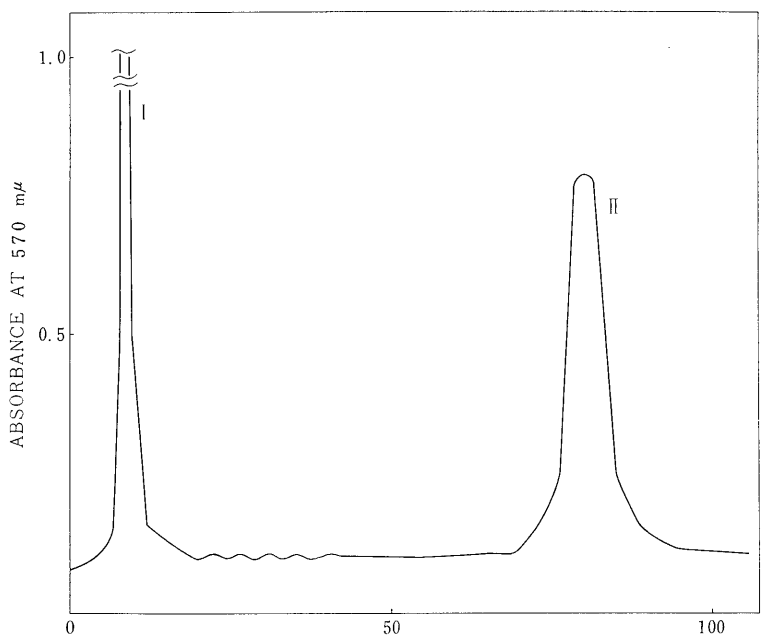
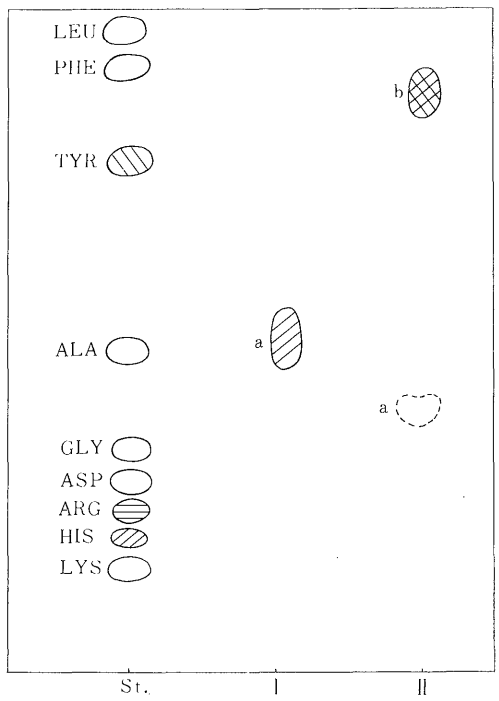


Fig. 5. Isolation of the peptic peptides from the tryptic peptide, α TVI-b. Column : Dowex 1 X2, 0.9 x 60 cm.

The effluent at each peak was dried under reduced pressure and purified by paper chromatography. Two main spots were observed in Fig. 6.



PEAK NUMBER IN FIG. 5

Fig. 6. Paper chromatogram of the peptic peptides from the tryptic peptide, α TVI-b.

Table II
Amino acid composition of the peptic peptides from the tryptic peptide, α TVI-b

	PI-a	PII-b
Lys	1.03	
His	0.97	1.00
Arg		
Cys		
Asp	1.02	
Thr		1.06
Ser	1.56	
Glu	1.09	
Pro		0.97
Gly	1.26	
Ala	1.14	
Val		
Met		
Ileu	0.90	
Leu	1.05	
Tyr		0.86
Phe		2.10

The amino acid composition of these peptide fragments is given in Table II.

(a) αTVI-b, P-I-a (Rf Leu 0.51)

	Lys	His	Asp	Ser	Glu	Gly	Ala	Ileu	Leu
composition	1.03	0.97	1.02	1.56	1.09	1.26	1.14	0.90	1.05
stage 1	n.d.	n.d.	0.00	1.69	1.08	1.15	1.18	0.89	0.99
2	n.d.	n.d.	0.00	1.67	1.12	1.15	1.21	0.85	0.12
3	n.d.	n.d.	0.00	1.07	0.96	1.06	1.07	1.09	0.00
4	n.d.	n.d.	0.00	1.10	0.99	1.04	1.01	0.90	0.00
5	n.d.	n.d.	0.00	1.12	1.08	0.10	1.01	0.81	0.00
6	n.d.	n.d.	0.00	0.10	1.11	0.00	1.03	0.85	0.00
7	n.d.	n.d.	0.00	0.00	1.11	0.00	0.14	0.89	0.00
8	n.d.	n.d.	0.00	0.00	0.23	0.00	0.00	1.00	0.00

The sequence was therefore Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Ileu-Lys.

→ → → → → → → →
P P P P P P P P

(b) αTVI-b, P-II-b (Rf Leu 0.90)

	His	Thr	Pro	Tyr	Phe
composition	1.00	1.06	0.97	0.86	2.10
stage 1	n.d.	**0.00**	0.91	1.00	2.08
2	n.d.	0.00	0.94	**0.00**	2.06
3	n.d.	0.00	1.00	0.00	**1.00**
4	n.d.	0.00	**0.00**	0.00	1.00
5	**n.d.**	0.00	0.00	0.00	1.00

The sequence was determined to be Thr-Tyr-Phe-Pro-His-Phe.

→ → → → →
P P P P P

By combining the above results, the entire sequence of Peptide α TVI-b was established as follows;

Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Ileu-Lys

|——— P-II-b ———→|←——————— P-I-a ———————→|

(13) α TVII-a (Rf Leu 0.35), α TVIII-b (Rf Leu 0.25)

These two peptides were mixed and then digested with pepsin. The digest was chromatographed on a column (0.9 x 60 cm) of Dowex 1X2 (200 to 400 mesh). Fig. 7 shows six peaks. The effluent at each

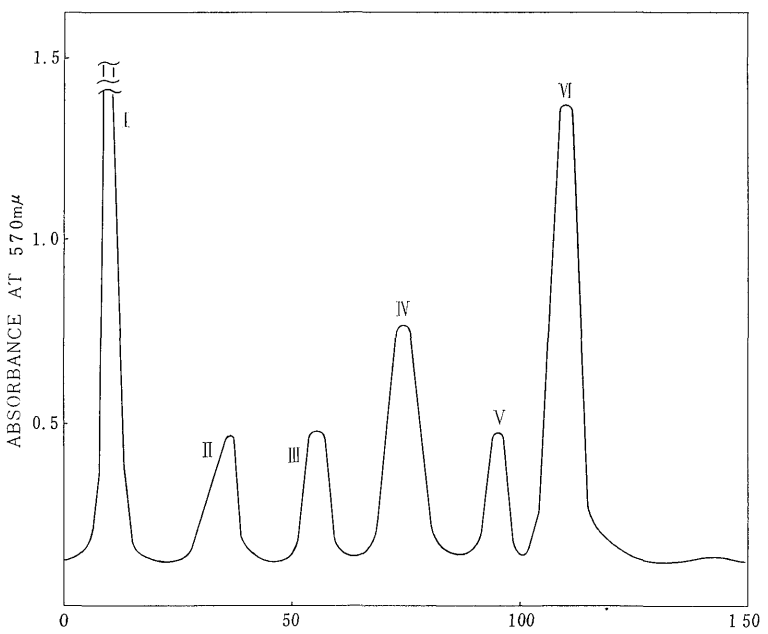
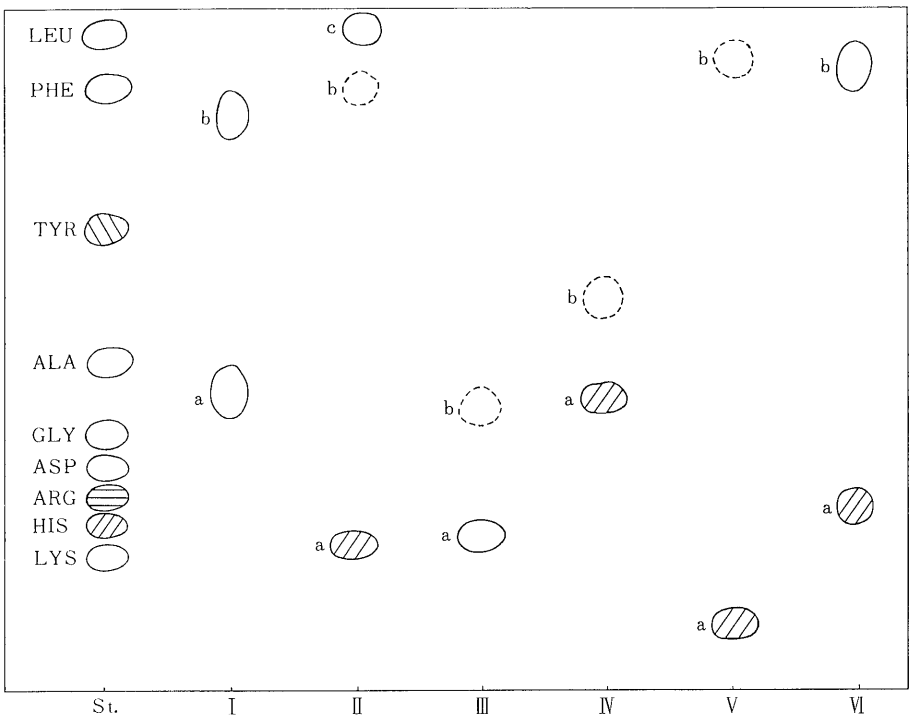


Fig. 7. Isolation of the peptic peptides from the tryptic peptide, α TVII-a. Column: Dowex 1 X2, 0.9 x 60 cm.



PEAK NUMBER IN FIG. 7

Fig. 8. Paper chromatogram of the peptic peptides from the tryptic peptide, α TVII-a.

Trble III Amino acid compositions of the peptic peptides from the tryptic peptide, α TVII-a

	Ia	Ib	IIa	IIc	IIIa	IVa	Va	VIa	VIb
Lys	1.01	1.02	1.10						
His			0.90			1.00	1.10	1.00	
Arg									
Cys									
Asp			1.83		1.02	1.00	2.01	3.10	0.90
Thr									0.96
Ser	1.31		1.92						
Glu			1.15			0.86		1.02	0.88
Pro									
Gly	0.92		1.03						
Ala	0.97	1.98	1.96	2.13	1.06	1.09	1.08	2.08	1.08
Val		1.10		0.86					
Met									
Ileu			1.04		0.91	1.04	0.80	1.81	1.93
Leu	0.79	0.90	1.04	1.01					
Tyr									
Phe									

peak was dried under reduced pressure and purified by paper chromatography. Nine main spots were observed in Fig. 8.
The amino acid composition of these peptide fragments is given in Table III.

(a) αTVII-a, P-I-a (Rf Leu 0.45)

This fragment was analyzed by the PTC method, suggesting that the sequence was Ser-Gly-Ala-Leu-Ser-Lys.

		Lys	Ser	Gly	Ala	Leu
		→ P	→ P	→ P	→ P	
composition		1.01	1.31	0.92	0.97	0.79
stage	1	n.d.	**0.08**	1.02	1.16	0.98
	2	n.d.	1.04	**0.00**	1.07	0.89
	3	n.d.	1.09	0.00	**0.10**	0.91
	4	n.d.	1.00	0.00	0.00	**0.15**

(b) αTVII-a, P-I-b (Rf Leu 0.83)

Analysis by the PTC method gave the following result, suggesting the sequence was Lys-Val-Ala-Leu-Ala.

		Lys	Ala	Val	Leu
		→ P	→ P	→ P	→ P
composition		1.02	1.98	1.10	0.90
stage	1	**n.d.**	2.01	1.05	0.92
	2	n.d.	1.88	**0.00**	1.12
	3	n.d.	**0.90**	0.00	1.10
	4	n.d.	1.00	0.00	**0.10**

(c) α TVII-a, P-III-a (Rf Leu 0.22)

The following result was obtained by the PTC method.

		Asp	Ala	Ileu
composition		1.02	1.06	0.91
stage	1	**0.21**	1.12	0.88
	2	0.10	**0.20**	1.00

The sequence was therefore Asn-Ala-Ileu.

(d) α TVII-a, P-IV-a (Rf Leu 0.43)

Analysis by the PTC method resulted as follows, suggesting the sequence was Ileu-Glu-His-Ala-Asp.

		His	Asp	Glu	Ala	Ileu
		→ P	→ P	→ P	→ P	
Composition		1.00	1.00	0.86	1.09	1.04
Stage	1	n.d.	**1.02**	0.84	1.18	**0.16**
	2	n.d.	0.80	**0.12**	1.20	0.00
	3	**n.d.**	0.97	0.00	1.03	0.00
	4	n.d.	1.00	0.00	**0.18**	0.00

(e) α TVII-a, P-V-a (Rf Leu 0.11)

The following result was obtained by the PTC method, suggesting the sequence was His-Ala-Asp-Asp-Ileu.

		→ P	→ P	→ P	→ P
		His	Asp	Ala	Ileu
composition		1.10	2.01	1.08	0.80
stage	1	n.d.	2.02	1.08	0.82
	2	n.d.	2.01	0.08	0.98
	3	n.d.	1.28	0.00	0.81
	4	n.d.	0.35	0.00	1.00

(f) αTVII-a, P-VI-b (Rf Leu 0.98)

The followiug result was obtained by the PTC method.

		Asp	Thr	Glu	Ala	Ileu
composition		0.90	0.96	0.88	1.08	1.93
stage	1	0.83	0.87	0.94	1.10	1.01
	2	0.90	0.00	0.89	1.05	1.01
	3	0.15	0.00	0.91	1.12	0.94
	4	0.03	0.00	0.93	0.08	1.05
	5	0.00	0.00	1.00	0.00	0.05

The sequence was therefore Ileu-Thr-Asn-Ala-Ileu-Glu.

→ P → P → P → P → P

As described above, the sequence of α TVII-a was determined. α TVII-a has lysine one residue more than α TVIII-b in the composition. This lysine that of α TII-b, and the tryptic digestion could not completely cleave the linkage between that lysine and α TVIII-b[15),24)]. Therfore, the entire sequence of α TVIII-b was as presented in Fig. 9.

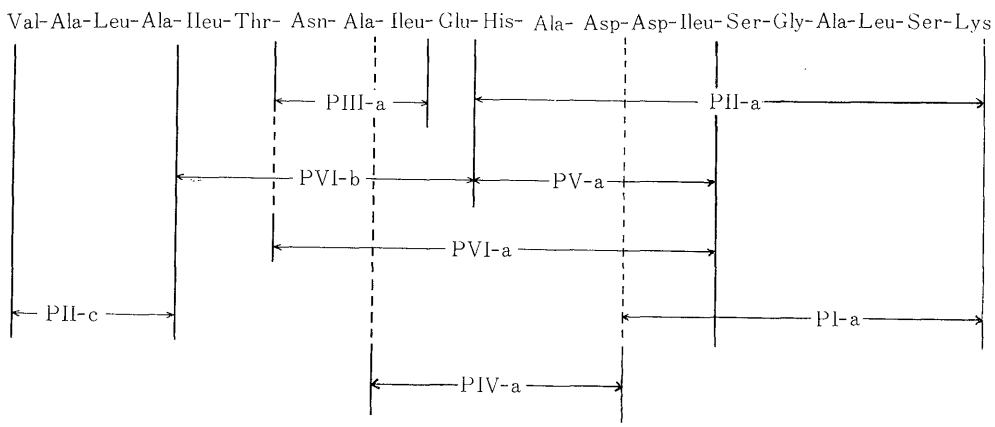


Fig. 9. Amino acid sequence in the tryptic peptide, α TVIII-b.

(14) αTIX-a (Rf Leu 0.23)

This peptide was further digested with pepsin, and then the digest was subjected to Dowex 1X2 column chromatography. As shown in

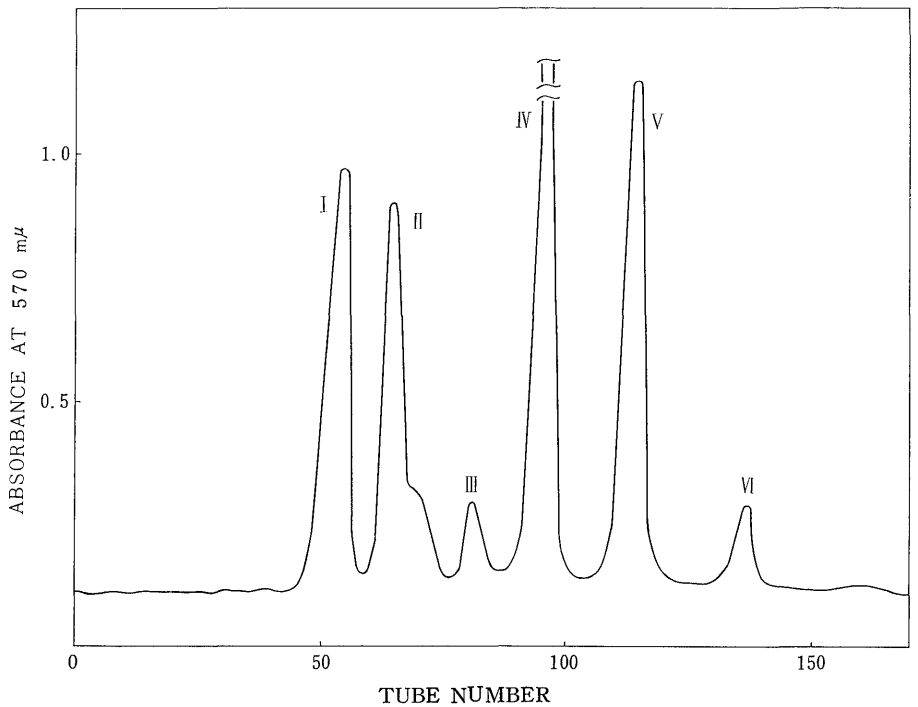


Fig. 10. Isolation of the peptic peptides from the tryptic peptide, αTIX-a.
Column: Dowex 1×2, 0.9×60cm.

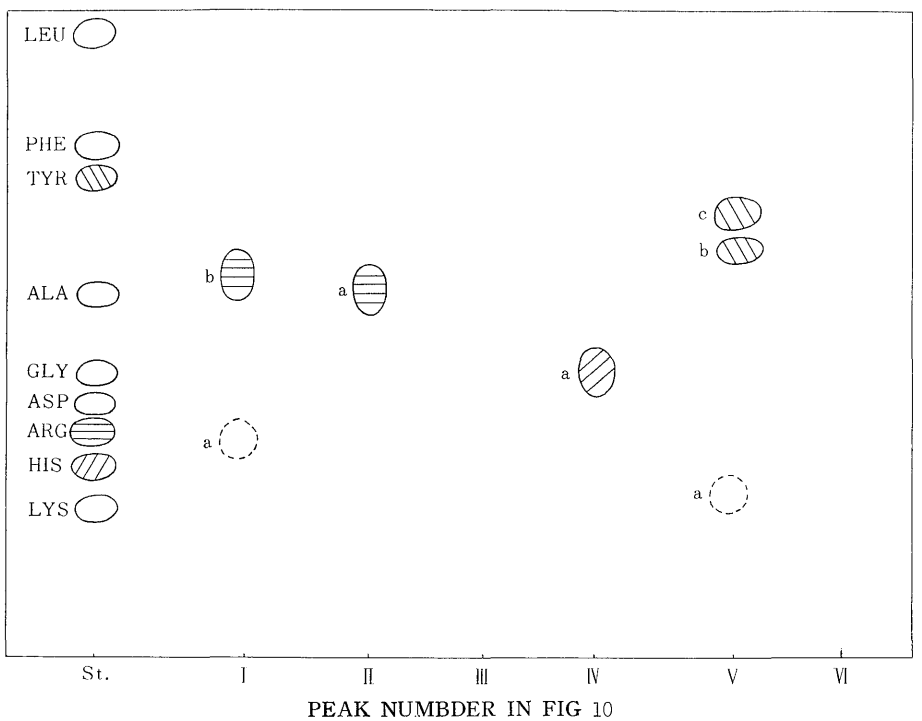


Fig. 11. Paper chromatogram of the peptic peptides from the tryptic peptide, αTIX-a.

Fig. 10, six peak were obtained. The effluent at each peak was dried under reduced pressure and then purified by paper chromatography. Five main spots were detected.
The amino acid composition of these peptide fragments is summarized in Table IV.

Table IV Amino acid compositions of the peptic peptides from the tryptic peptide, α TIX-a

	Ib	IIa	IVa	Vb	Vc
Lys					
His			0.99		
Arg	0.98	0.95			
Cys					
Asp					
Thr		0.84		0.88	
Ser					
Glu	1.03	1.09	1.81	1.15	1.05
Pro					
Gly			1.08	1.13	1.13
Ala			2.11	1.12	1.05
Val					
Met					
Ileu			0.96		
Leu	1.09	1.05			
Tyr				0.71	0.75
Phe					

(a) α TIX-a, P-II-a (Rf Leu 0.59)

The following result was obtained by the PTC method, suggesting the sequence was Thr-Leu-Glu-Arg.

		Arg	Thr	Glu	Leu
		→ P	→ P		
composition		0.95	0.84	1.09	1.05
stage	1	n.d.	**0.00**	1.04	0.96
	2	n.d.	0.00	1.00	**0.06**

(b) α TIX-a, P-IV-a (Rf Leu 0.43)

This fragment was analyzed by the PTC method as follows.

		His	Glu	Gly	Ala	Ileu
composition		0.99	1.81	1.08	2.11	0.96
stage	1	n.d.	2.05	1.04	1.90	**0.00**
	2	n.d.	2.00	0.94	**1.05**	0.00
	3	n.d.	2.01	**0.26**	0.96	0.00
	4	**n.d.**	1.90	0.00	1.03	0.00
	5	n.d.	2.00	0.00	**0.00**	0.00

The sequence was determined to be Ileu-Ala-Gly-His-Ala-Glu-Glu.

$\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$

(c) α TIX-a, P-V-b (Rf Leu 0.68)

Analysis by the PTC method gave the following result, suggesting the sequence was Tyr-Gly-Ala-Glu-Thr.

$\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$

	Thr	Glu	Gly	Ala	Tyr
composition	0.88	1.15	1.13	1.12	0.71
stage 1	0.82	1.08	1.05	1.04	**0.00**
2	0.83	1.11	**0.00**	1.05	0.00
3	0.92	1.08	0.00	**0.00**	0.00
4	1.00	**0.08**	0.00	0.00	0.00

By combining the above results from the peptide fragments together, the entire sequence of α TIX-a was determined as shown in Fig. 12.

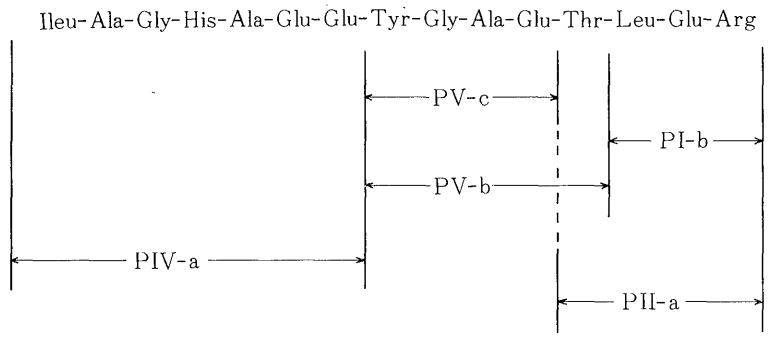


Fig. 12. Amino acid sequence in the tryptic peptide, αTIX-a

8) *Determination of the N-Terminal Residue of the Insoluble Tryptic Peptide by the DNP Method.*

The N-terminal residue of the insoluble tryptic peptide was known to be leucine by the DNP method.

9) *Column Chromatography, Paper Chromatography, and Amino Acid Composition of Peptic Peptides from the Insoluble Tryptic Peptide.*

Five peaks are observed in Fig. 13. The effluent at each peak was dried under reduced pressure and purified by paper chromatography. As shown in Fig. 14, eight main spots were obtained. The amino acid composition of these spots is given in Table V.

10) *Amino Acid Sequence of the Peptic Peptides from the Insoluble Tryptic Peptide*

(1) P-I-b (Rf Leu 0.36)

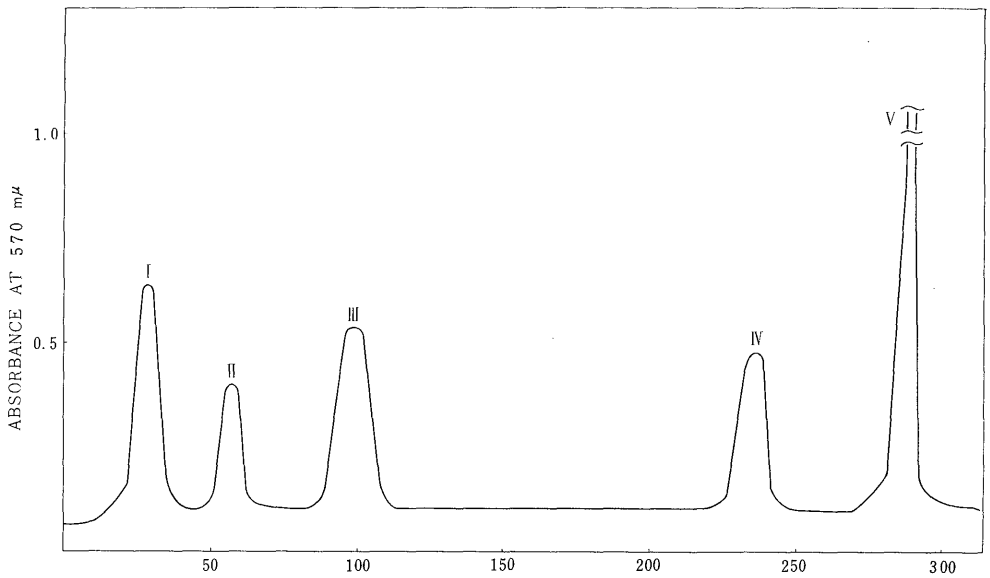


Fig. 13. Isolation of the peptic peptides from the insoluble tryptic peptide

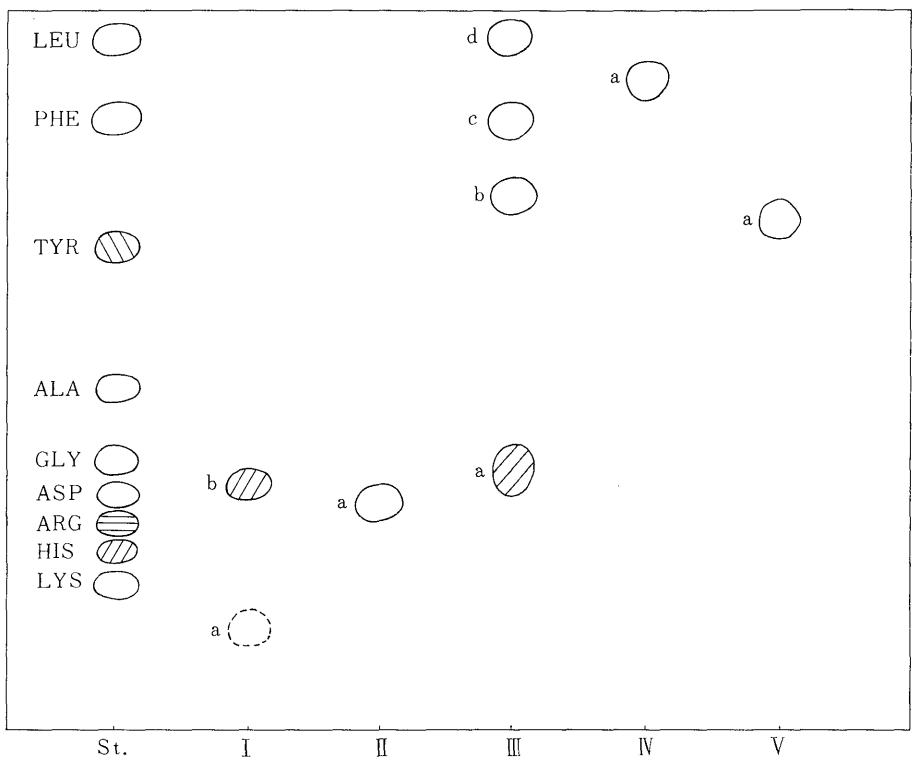


Fig. 14. Paper chromatogram of the peptic peptides from the insoluble tryptic peptide.

Table V Amino acid compositions of the peptic peptides from the insoluble tryptic peptide

	Ib	IIa	IIIa	IIIb	IIIc	IIId	IVa	Va
Lys	0.88	1.01						1.01
His	1.12		1.03					
Arg								
Cys							+	+
Asp								1.01
Thr		0.96			0.96			0.83
Ser				0.96				
Glu			0.89				1.08	
Pro	0.97		1.05					
Gly					1.05		1.06	1.17
Ala	2.03	1.04	2.03					1.16
Val	0.98		0.97		1.98	2.25		1.96
Met								
Ileu								
Leu	1.07		1.03	1.04	1.01	1.85	1.94	1.97
Tyr								
Phe							0.90	0.89

Analysis by the PTC method resulted as follows.

	Lys	His	Pro	Ala	Val	Leu
composition	0.88	1.12	0.97	2.03	0.98	1.07
stage 1	0.98	1.02	0.95	2.08	0.99	**0.08**
2	0.96	1.03	1.07	**1.01**	0.92	0.00
3	0.98	1.03	**0.18**	1.00	0.93	0.00
4	**0.10**	0.98	0.00	1.00	0.92	0.00
5	0.00	0.99	0.00	1.00	**0.10**	0.00
6	0.00	**0.15**	0.00	1.00	0.00	0.00

The sequence was therefore determined to be Leu-Ala-Pro-Lys-Val-His-Ala.

	→	→	→	→	→	→
	P	P	P	P	P	P

(2) P-II-a (Rf Leu 0.32)

The following result was obtained by the PTC method, suggesting the sequence was Thr-Ala-Lys.

	→	→	
	P	P	
	Lys	Thr	Ala
composition	1.01	0.96	1.04
stage 1	n.d.	**0.12**	1.00
2	n.d.	0.00	**0.15**

(3) P-III-a (Rf Leu 0.37)

This peptide fragment was analyzed by the PTC method as follows.

	His	Glu	Pro	Ala	Val	Leu
composition	1.03	0.89	1.05	2.03	0.97	1.03
stage 1	n.d.	0.91	1.04	2.04	**0.05**	1.02
2	n.d.	0.92	1.05	**1.03**	0.00	1.01
3	**n.d.**	0.88	1.05	**1.03**	0.00	0.85
4	n.d.	0.95	0.91	1.02	0.00	**0.10**
5	n.d.	0.94	**0.11**	**1.06**	0.00	0.00
6	n.d.	1.00	0.00	**0.16**	0.00	0.00

The sequence was therefore determined to be Val-Ala-His-Leu-Pro-Ala-Glu.

	→	→	→	→	→	→
	P	P	P	P	P	P

(4) P-III-b (Rf Leu 0.78)

The N-terminal amino acid was known to be serine by the DNP method, suggesting the sequence was Ser-Leu.

→
D

(5) P-III-c (Rf Leu 0.86)

Analysis by the PTC method resulted as follows.

	Thr	Gly	Val	Leu
composition	0.96	1.05	1.98	1.01
stage 1	0.98	1.04	**1.01**	1.02
2	0.96	**0.13**	1.02	1.02
3	**0.08**	0.00	0.98	1.01
4	0.00	0.00	**0.15**	1.00

The sequence was therfore determined to be Val-Gly-Thr-Val-Leu

	→	→	→	→
	P	P	P	P

(6) P-III-d (Rf Leu 1.00)

The following result was obtained by the PTC method.

	Val	Leu
composition	2.25	1.85
stage 1	2.03	**0.97**
2	**1.06**	0.94
3	**0.18**	1.00

The sequence was therefore Leu-Val-Val-Leu.

	→	→	→
	P	P	P

(7) P-IV-a (Rf Leu 0.92)

Analysis by the PTC method resulted as follows.

	Cys	Glu	Gly	Leu	Phe
composition	+	1.08	1.06	1.94	0.90
stage 1	+	1.09	0.97	**0.99**	0.93
2	+	1.10	1.01	**0.11**	0.89
3	+	1.04	**0.10**	0.00	0.96

4	+	**0.28**	0.00	0.00	1.00
5	−	0.00	0.00	0.00	1.00

The sequence was therefore Leu-Leu-Gly-Gln-Cys-Phe.

$\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$

(8) P-V-a (Rf Leu 0.75)

	Lys	Cys	Asp	Thr	Gly	Ala	Val	Leu	Phe
composition	1.01	+	1.01	0.83	1.17	1.16	1.96	1.97	0.89
stage 1	n.d.	+	**0.05**	0.90	1.15	1.10	1.98	1.98	0.91
2	**n.d.**	+	0.00	0.89	1.16	1.09	1.99	2.01	0.92
3	n.d.	+	0.00	0.91	1.13	1.09	1.98	2.00	**0.08**
4	n.d.	+	0.00	0.91	1.10	1.11	1.97	**1.01**	0.00
5	**n.d.**	−	0.00	0.88	1.08	1.06	1.98	1.02	0.00
6	n.d.	−	0.00	0.90	1.06	**0.10**	1.98	1.02	0.00
7	n.d.	−	0.00	0.91	1.05	0.00	**1.03**	1.01	0.00

The above result obtained by the PTC method suggests the sequence was Asp-Lys-Phe-Leu-Cys-Ala-Val-(Gly, Thr, Val, Leu).

$\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$ $\overrightarrow{P}$

Since Peptide P-III-c corresponds to the C-terminal portion of this fragment, the sequence of P-V-a was determined to be Asp-Lys-Phe-Leu-Cys-Ala-Val-Gly-Thr-Val-Leu.

By combining the above results together, the whole sequence of the insoluble tryptic peptide was determined as shown in Fig. 15.

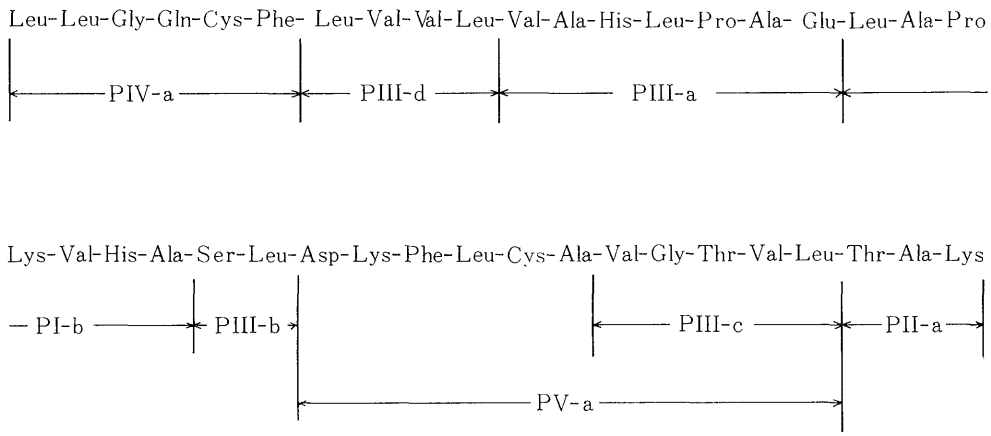


Fig. 15. Amino acid sequence in the insoluble tryptic peptide.

DISCUSSION

Concerning the the components of adult white leghorn hemoglobin, FASNER,[18] SAHA,[42] and HUSIMAN[48] reported that the hemoglobin consisted of two kinds of components. However, MATSUDA[32] and D'AMELO[16] proposed that it was composed of three kinds of compnents. MANWELL[28]

stated in his report that the older hemoglobin samples often showed a broadeding of the acidic minor component hemoglobin zone, frequently showing subdivision into two or more diffuse bands. This phenomenon was also observed in the present experiment. However, it is yet unknown why the number of the components differs thus among investigators.

It turned out by the present experiment that adult white leghorn hemoglobin contained two residues of cysteine in the α polypeptide chain. This fact is in agreement with the report of SAHA et al.[43] on the amino acid composition. However, the estimation of cysteine resulted in 1.40 mole per one molecule of the α polypeptide chain, probably because the α polypeptide chain had not dissolved completely in the process of the oxidation with performic acid. Incidentally, no hemoglobins so far studied contain two or more cysteine residues in their α polypeptide chains.

As for tryptophan residues, the estimation resulted in 0.14 mole per one molecule of the α polypeptide chain. According to KOSHLAND et al.[3] this result indicates that no residue of tryptophan was contained in the α polypepide chain of adult white leghorn hemoglobin. In addition, no tryptophan residues were found in the process of the sequence determination. HILSMANN et al.[11] stated in their paper on the fragments from the α polypeptide chain of llama hemoglobin that the tryptophan residue at Position 14 in human hemoglobin was replaced by Phenylalanine in llama hemoglobin.

Tadle VI Amino acid sequence in the soluble tryptic peptides from the α polypeptide chain of AII component of adult white leghorn hemoglobin.

peptide	amino acid sequence
αTI-a	Leu-Arg
αTII-a	Gly-His-Gly-Lys
αTII-b	Lys
αTIII-b	Asn-Asn-Val-Lys
αTIII-c	Tyr-Arg
αTIII-d	Met-Phe-Ileu-Gly-Phe-Pro-Thr-Thr-Lys
αTIV-a	Val-Leu-Ser-Asn-Ala-Asp-Lys
αTIV-d	Gly-Ileu-Phe-Thr-Lys
αTV-a	Leu-Ser-Asp-Leu-His-Ala-His-Lys
αTV-b	Val-Asp-Pro-Val-Asn-Phe-Lys
αTVI-a	Val-His-Ala-Ser-Leu-Asp-Lys
αTVI-b	Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Ileu-Lys
αTVIII-b	Val-Ala-Leu-Ala-Ileu-Thr-Asn-Ala-Ileu-Glu-His-Ala-Asp-Asp-Ileu-Ser-Gly-Ala-Leu-Ser-Lys
αTIX-a	Ileu-Ala-Gly-His-Ala-Glu-Glu-Tyr-Gly-Ala-Glu-Thr-Leu-Glu-Arg

The amino acid sequences in all the tryptic peptides from the α polypeptide chain of adult white leghorn hemoglobin was established by the present investigation as shown in Table VI.

These results are discussed by comparing with the known sequence of human hemoglobin. First, it is known that the sequeuce in the five tryptic peptides, α TI-a, α TII-a, α TII-b, α TIII-c, and α TV-b are quite similar to those in α T10, α T7, α T8, α T14, and α T11 of human hemoglabin, respectively. Peptide α TIV-d seems to correspond to α T3 of human hemoglobin, however, in the sequence analysis, four of the five residues are different from each other. This peptide was in high yield and occupied constantly the same position both on the column and paper chromatograms, suggesting that it is not a contamination. Peptide α TV-a has quite the same sequence with Position 83 to 90 in the α polypeptide chain of human hemoglobin, which accounts for the C-terminal portion of α T9. Peptide α TVI-a has the identical sequence with Position 121 to 127 in the α polypeptide chain of huma hemoglobin, which accounts for the C-terminal portion of α T12. Similarly, αTIII-b, α TIII-d, α TIV-a, α TVI-b, α TVIII-b, and α TIX-a are presumed to correspond to α T2, α T5, α T1, α T6, the N-terminal portion of α T9, and α T4, respectively.

BRAUNITZER et al. [10,15], in their study on human hemoglobin, obtained the "core" peptide in which Lys-Phe linkage was not cleaved, whereas KONIGSBERG et al.[20),24)] succeeded in cleaving this linkage. In the present investigation were obtained both kinds of these peptides, that is, αTVI-a in which Lys-Phe linkege was cleaved, and the α core P-V-a in which the linkage was not cleaved.

In the PTC degradation method, removal of glycine, asparagine, and aspartic acid residues was not successful. KONIGSBERG et al.[49)] also described about this fact and presumed that it was probably because the formation of phenylisocarbamyl peptides prevented them from cyclization.

PERUTZ[37)] and BRAUNITZER et al.[7)] brought forward a hypothesis that there exist eight invariable residues common to hemoglobins from various kinds of species and that they were glycine at B6, proline at C2, phenylalanine at CD1, histidine at E7, leucine at F4, histidine at F8, lysine at H10, and tyrosine at H23. It was confirmed by the present investigation that amino acid replacement was not observed at the above-mentioned eight positions in white leghorn hemoglobin.

Furthermore, PERUTZ[37)] proposed the rule of isopolar substitution that 33 positions of the internal site and 10 positions of the surface cervices were occupied by non-polar residues. In application of this rule to the results obtained by the present investigation, the amino acid substitutions in the internal site between human and white leghorn hemoglobin which were found at seven positions, A11, A12, A15, B9, E4, E15, and E19 were all among non-polar residues with the exception

of B9, which was a non-polar residue, alanine, in human hemoglobin but apolar-residue. threonine, in white leghorn hemoglobin. This is considered to be one of the three exceptions pointed out by PERUTZ et al.[37], who stated that in some cases B9 was occupied by threonine or serine which was probably hydrogen-bonded internally.

Concerning the correlation between amino acid substitution and gene, BEAL et al.[5] explained that one amino acid substitution in abnormal human hemoglobin was caused by single base change in the triplet coden in gene which controls hemoglobin biosynthesis. According to the amino acid triplet coden proposed by NIERENBERG[35], 21 of 35 amino acid substitutions in the α polypeptide chain between human and white leghorn hemoglobins, that is, the amino acid substitutions at Positions 8, 12, 17, 18, 19, 22, 28, 34, 55, 66, 70, 71, 73, 76, 102, 103, 105, 110, 117, 118, and 133, are presumed to be due to single base change in the triplet code; the remaining 14 amino acid substitutions, that is, those at Position 4, 13, 14, 15, 35, 64, 77, 78, 82, 108, 120, 130, 131, and 138 are presumed to be due to double base change.

FLORKIN[19] stated that according as animals evolve higher, their hemoglobins in general contain less histidine and tryptophan and that hemoglobins of higher animals contain almost no isoleucine and few methionine. It turned out by the present investigation that white leghorn hemoglobin contained seven isoleucine residues, whereas human hemoglobin has no isoleucine residues. However, the former has histidine, methionine, and tryptophan one residue less than the latter. This fact is contrary to the above-described FLORKIN's view.

CONCLUSION

The sequence in all the tryptic peptides from the α polypeptide chain of adult white leghorn hemoglobin was determined. The results were discussed in comparison with the known sequence in the α polypeptide chain of human hemoglobin. Amino acid substitution was found at 35 positions between the two hemoglobins. Furthermore, it is confirmed that these substitutions are compatible with PERUTZ's view of eight invariable residues in hemoglobins as well as to his rule of non-polar substitution.

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